A METHOD FOR ISOLATION OF CHROMOSOMAL AND PLASMID DNA FROM *YERSINIA ENTEROCOLITICA* FOR SIMULTANEOUS AMPLIFICATION BY POLYMERASE CHAIN REACTION: A POSSIBLE MODEL FOR OTHER BACTERIA

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ABSTRACT

A commercial DNA isolation kit was evaluated for simultaneous isolation of chromosomal and plasmid DNA from Yersina enterocolitica *for polymerase chain reaction amplification. The genomic and plasmid DNA samples obtained by use of the kit were suitable for use in polymerase chain reactions both individually and in multiplex reactions. The results obtained by the use of the kit suggest that this kit may be applied to isolate both genomic and plasmid DNA from other microorganisms for polymerase chain reaction amplification.*

INTRODUCTION

The polymerase chain reaction (PCR), which has the capacity to amplify specific sequences of DNA has been successfully developed as a specific and sensitive diagnostic method for the detection of microorganisms in aquatic environments, food or dairy products and clinical samples (Belák and Ballagi-Pordány 1993; Swaminathan and Feng 1994; Vanbelkum 1994). Since direct application of PCR to complex substrates results in a lack of amplification products or poor sensitivity, bacterial DNA extraction must be performed to avoid PCR inhibition due to com-

ponents present in food, clinical and environmental samples (Lantz *et al.* 1994). Sample treatment normally involves the purification of DNA from isolated cells or from the sample as a whole. Since target gene(s) of the pathogenic and non-pathogenic strains may be located either in the chromosome (Baez and Juneja 1995; Bhaduri *et al.* 1986), plasmids (Andersen and Omiecinski 1992) or in both (Bhaduri 1993, 1994; Koeppel *et al.* 1993; Kwaga *et al.* 1992; Miller *et al.* 1989; Nakajima *et al.* 1992), two different techniques are often employed for the isolation of chromosomal and plasmid DNA for PCR amplification (Kwaga *et al.* 1992; Nakajima *et al.* 1992). Several commercial kits are successfully used for extraction of genomic and plasmid DNA for PCR amplification (Andersen and Omiecinski 1992; Baez and Juneja 1995). But none of these kits have been evaluated for simultaneous isolation and subsequent PCR amplification of both genomic and plasmid DNA. In situations where time and complexity are prime considerations, it would be advantageous to simultaneously isolate both genomic and plasmid DNAs for PCR using a single method.

The present study was designed to evaluate a commercial DNA extraction kit for simultaneous isolation of both genomic and plasmid DNA for PCR amplification.

MATERIALS AND METHODS

Bacteria

Yersinia enterocolitica (strain GER; serotype O:3) was used as a model system for a plasmid-bearing virulent bacterium. This bacterium was chosen for the evaluation of a DNA extraction procedure for the simultaneous isolation of genomic and plasmid DNA because the genetic loci that confer pathogenicity have been identified on both the chromosome and plasmid (Bhaduri 1993, 1994; Koeppel *et al.* 1993; Kwaga *et al.* 1992; Miller *et al.* 1989; Nakajima *et al.* 1992). A detailed description of the strain, source, preparation of inoculum and incubation conditions is given elsewhere (Bhaduri 1993).

DNA Extraction Procedures

The plasmid-bearing virulent strain of *Y. enterocolitica* was grown in 5 ml of brain heart infusion (Difco Laboratories, Detroit, MI) broth overnight (18–24 h) at 28C to a population density of approximately 10^9 cfu/ml. Cells were harvested by centrifugation at 10,400 g (4C, 10 min) and washed twice with phosphate buffered saline (0.01 M sodium phosphate-0.85% saline, pH 7.5). DNA extraction was performed using the G-Nome™ kit from BIO 101 (La Jolla, CA) by the following protocol:

(1) The washed pellet was resuspended by vortexing in 231 μl of *Cell Suspension Solution* until the suspension appeared homogeneous.

(2) 6.25 μl of *RNase Mixx* was added and mixed thoroughly by gentle vortex mixing.

(3) 12.50 μl of *Cell Lysis/Denaturing Solution* was added and mixed thoroughly by vortexing.

(4) The suspension was incubated at 55C for 15 min to produce an almost clear viscous consistency.

(5) 3.13 μl of *Protease Mixx* was added and mixed thoroughly by gentle vortex mixing.

(6) The suspension was incubated at 55C for 90 min.

(7) Addition of 62.50 μl of "Salt Out" Mixture, followed by gentle vortex mixing caused the appearance of a white flocculent material. The suspension was chilled in an icebath for 10 min to allow precipitation.

(8) Centrifugation for 10 min at maximum speed in an Eppendorf centrifuge caused the salt-precipitated complex to form a white pellet. The supernatant fluid was collected carefully, avoiding the pellet. If a precipitate remained in the supernatant fluid, the suspension was again centrifuged until it was clear.

(9) The supernatant fluid was transferred to a 2 ml Eppendorf tube. After measuring the volume of the supernatant fluid, the DNA was precipitated by adding sodium acetate to a final concentration of 0.3 M, followed by addition of 2.5 volumes of chilled ethanol. The mixture was held overnight at −20C to allow complete DNA precipitation. Precipitated DNA was recovered by centrifugation in an Eppendorf centrifuge at 4C for 30 min. The supernatant fluid was decanted and residual ethanol was removed under a stream of nitrogen.

(10) DNA was dissolved in 50 μl of sterile distilled water. The DNA concentration of the samples was determined by absorbance at 260 nm and the purity of the DNA evaluated by The A_{260}/A_{280} ratio (Bhaduri *et al.* 1980). The DNA samples were stored at −20C until they were used for PCR amplification.

Selection of PCR Primers

One of the genetic loci that confers the pathogenicity of *Y. enterocolitica* has been identified as the *ail* gene (attachment-invasion locus) on the chromosome (Miller *et al.* 1989). All isolates of *Y. enterocolitica* that show virulence in humans contain DNA sequences homologous to the *ail* locus, whereas avirulent environmental isolates do not (Miller *et al.* 1989). Virulence in *Y. enterocolitica* is also associated with the presence of a 67–70 kilobase plasmid (Bhaduri 1993, 1994; Doyle and Cliver 1990; Kapperud 1991; Kwaga and Iversen 1991). A key regulatory gene, *virF*, present on the virulence plasmid encodes a transcriptional activator for the expression of plasmid-encoded outer membrane protein *yop*51 (Cornelis *et al.* 1989). Recently, a method for the rapid specific detection of pathogenic strains

of *Y. enterocolitica* by PCR was reported by Nakajima *et al.* (1992). They used primers based on the *ail* gene and *virF* gene, respectively. The primers used by us for the *ail* and *virF* genes of pathogenic *Y. enterocolitica* were those previously reported (Nakajima *et al.* 1992). The primers (5′-ACTCGATGATAACTGGGGAG-3′ and 5′-CCCCCAGTAATCCATAAAGG-3′) for detection of the *ail* gene (664- to 833-nucleotide region) amplified a 170 base pair (bp) DNA fragment from the chromosome. The primers (5′-TCATGGCAGAACAGCAGTCAG-3′ and 5′-ACTCATCTTACCATTAAGAAG-3′) for detection of the *virF* gene (430- to 1020-nucleotide region) amplified a 591 bp product from the virulence plasmid. Each primer set for the *ail* or *virF* gene was used for the detection of each gene, and a mixture of primers against the *ail* and *virF* genes was used for the detection of both genes in multiplex reaction. The oligonucleotide primers utilized in this study were synthesized by the Appligene Company (Pleasanton, CA).

PCR Amplification

PCR was performed essentially as described by Nakajima *et al.* (1992). The GeneAmp PCR reagent kit with AmpliTaq DNA polymerase was purchased from Perkin-Elmer Cetus Corporation (Norwalk, CT). The standard reaction mixtures (50 μl) contained 5 μl template DNA sample, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.001% (wt/vol.) gelatin, 100 μl each deoxynucleoside triphosphate, 0.2 μM *ail* and/or *virF* primers and 0.5 U of AmpliTaq DNA polymerase. The samples were amplified by PCR with the programmable heating block incubator (GeneAmp PCR system 9600, Perkin-Elmer Corporation), which consisted of the following: predenaturation at 94C for 1 min, 30 cycles of denaturation at 94C for 0.5 min, primer annealing at 55C for 1 min and extension at 70C for 2 min, followed by further extension at 70C for 5 min. A negative control with all of the reaction components except template DNA was included with each test run. After PCR amplification, 5 μl of endostop solution (50% glycerol-0.02% bromophenol blue-60 mM EDTA, pH 8.0) was added and 20 μl of each PCR product was analyzed by electrophoresis on a 2.0% agarose gel at a constant voltage of 80 V in TAE buffer (40 mM Tris-acetate-1 mM EDTA, pH 8.0) for 60 min (Bhaduri *et al.* 1980). The amplified DNA fragments were visualized by ethidium bromide (0.5 μg per ml) staining and UV transillumination (at 302 nm) of the gel (Bhaduri *et al.* 1980). Photographs of the stained gel under UV light were made for permanent records.

RESULTS AND DISCUSSION

The usefulness of the PCR for the detection and identification of bacteria in different types of samples can be limited by the presence of substances that in-

hibit the PCR. Thus, the extraction of DNA to remove PCR-inhibitory substances from samples must be carried out to reach an acceptable detection level for routine analysis. In this work, a DNA extraction procedure was investigated for the isolation of both genomic and plasmid DNA for PCR amplification from plasmid-bearing cells of *Y. enterocolitica*. DNA isolated by the procedure as described in "MATERIALS AND METHODS" section was subjected to PCR amplification. Each primer pair for the *ail* or *virF* genes was confirmed to amplify a 170 bp product from the chromosome or a 591 bp product from the virulence plasmid (Fig. 1, lanes 1 and 2, respectively). With a mixture of *ail* and *virF* primers, PCR could amplify genomic and plasmid DNA in a multiplex reaction (Fig. 1, lane 3). Thus, the DNA samples obtained by this extraction procedure contained both genomic and plasmid DNA and were suitable for use in polymerase chain reaction amplification. These results were similar to those reported by previous investigators using separate extraction methods for genomic and plasmid DNA

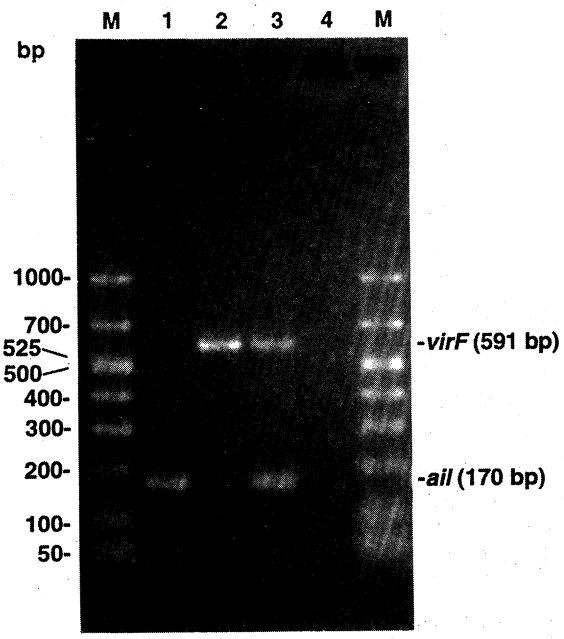

FIG. 1. SPECIFIC AMPLIFICATION OF 170-, AND 591 BP PRODUCTS BY PCR

Lanes: 1, 170 bp product from chromosome with *ail* primers; 2, 591 bp product from virulence plasmid with *virF* primers; 4, 170- and 591 bp products with mixture of both *ail* and *virF* primers from chromosome and virulence plasmid respectively; 4, negative control with no template; M, 50 to 1000 bp ladder marker.

(Koeppel *et al.* 1993; Kwaga *et al.* 1992; Nakajima *et al.* 1992). The DNA preparation kit used in this study eliminates many of the time consuming and labor intensive steps involved in two separate DNA extraction procedures required for genomic and plasmid DNA, yet maintains the yield and product purity which traditional methods provide. This method provides a fast and simple method for the simultaneous isolation of chromosomal and plasmid DNA templates from bacteria intended for PCR amplification. Further studies are in progress to determine the applicability of this technique for the detection of microorganisms in aquatic, environmental, food and clinical samples.

REFERENCES

ANDERSEN, M.R. and OMIECINSKI, C.J. 1992. Direct extraction of bacterial plasmids from food for polymerase chain reaction amplification. Appl. Environ. Microbiol. *58*, 4080–4082.

BAEZ, L.A. and JUNEJA, V.K. 1995. Detection of enterotoxigenic *Clostridium perfringens* in raw beef by polymerase chain reaction. J. Food Protec. *58*, 154–159.

BELÁK, S. and BALLAGI-PORDÁNY, A. 1993. Experience on the application of the polymerase chain reaction in a diagnostic laboratory. Molecular and Cellular Probes, *7*, 241–248.

BHADURI, S. 1993. Simple and rapid methods for identification of virulent plasmid-bearing clones of *Yersinia enterocolitica*. J. Rapid Methods and Automation in Microbiol. *2*, 27–38.

BHADURI, S. 1994. Use of multiple plasmid-associated virulence determinants for identification of pathogenic *Yersinia enterocolitica*. J. Rapid Methods and Automation in Microbiol. *3*, 97–103.

BHADURI, S., DEMCHICK, P.H. and HUHTANEN, C.N. 1986. Heterogeneity in the cellular protein profiles of *Clostridium botulinum* types A and B. J. Ind. Microbiol. *1*, 165–170.

BHADURI, S., KASAI, T., SCHLESSINGER, D. and RASKAS, H.J. 1980. pMB9 plasmids bearing the *Salmonella typhimurium his*operon and *gnd* gene. Gene *8*, 239–253.

CORNELIS, G., SLUITERS, C., de ROUVROIT, C. and MICHIELS, T. 1989. Homology between *virF*, the transcriptional activator of the *Yersinia* virulence regulon, and *ara*C, the *Escherichia coli* arabinose operon regulator. J. Bactriol. *171*, 254–262.

DOYLE, M.P. and CLIVER, D.O. 1990. *Yersinia enterocolitica*. In *Foodborne Diseases*, (D.O. Cliver, ed.) pp. 223–228, Academic Press, San Diego, CA.

KAPPERUD, G. 1991. *Yersinia enterocolitica* in food hygiene. Int. J. Food Microbiol. *12*, 53–66.

KOEPPEL, E., MEYER, R., LUETHY, J. and CANDRIAN, U. 1993. Recognition of pathogenic *Yersinia enterocolitica* by crystal violet binding and polymerase chain reaction. Lett. Appl. Microbiol. *17*, 231–234.

KWAGA, J.K.P. AND IVERSEN, J.O. 1991. Laboratory investigation of virulence among strains of *Yersinia enterocolitica* and related species from pigs and pork products. Can. J. Microbiol. *38*, 92–97.

KWAGA, J.K.P., IVERSEN, J.O. and MISRA, V. 1992. Detection of pathogenic *Yersinia enterocolitica* by polymerase chain reaction and digoxigenin-labeled polynucleotide probes. J. Clin. Microbiol. *30*, 2668–2673.

LANTZ, P.-G., HANHN-HÄGERDAL, B. and RÅDSTR¾OM, P. 1994. Sample preparation methods in PCR-based detection of food pathogens. Trends in Food Sc. and Technol. *5*, 384–389.

MILLER, V.L., FARMER, J.J. III, HILL, W.E. and FALKOW, S. 1989. The *ail* locus is found uniquely in *Yersinia enterocolitica* serotypes commonly associated with disease. Infec. Immun. *57*, 121–131.

NAKAJIMA, H., INOUE, M., MORI, T., ITOH, K-I., ARAKAWA, E. and WATANABE, H. 1992. Detection and identification of *Yersinia pseudotuberculosis* and pathogenic *Yersinia enterocolitica* by an improved polymerase chain reaction method. J. Clin. Microbiol. *30*, 2484–2486.

SWAMINATHAN, B. and FENG, P. 1994. Rapid detection of food-borne pathogenic bacteria. Annu. Rev. Microbiol. *48*, 401–426.

VANBELKUM, A. 1994. DNA fingerprinting of medically important microorganisms by use of PCR. Clin. Microbiol. Rev. *7*, 174–184.